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# The proliferative actions of insulin, insulin-like growth factor-I, epidermal growth factor, butyrate and propionate on ruminal epithelial cells in vitro

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**Abstract**

The rumen epithelium increases in size and surface area in response to increased feed intake, presumably due to increased epithelial cell proliferation. Isolated ruminal cells were cultured in vitro to determine the effect of various growth factors on ruminal cell proliferation. The growth factors evaluated included insulin, insulin-like growth factor-I and epidermal growth factor. VFA propionate and butyrate were also evaluated. Ruminal epithelial cells were isolated from five lambs via serial tryptic digestion and prepared for sterile culture by serial washes in sterile buffered salts solution. Cell proliferation was assessed by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetra-zoliumbromide] (MTT) assay. Cell growth was stimulated to 75, 96 and 97% of the growth of cells cultured in DMEM supplemented with 5% FCS (for Ins, IGF-I and EGF, respectively). Propionate and butyrate addition resulted in an overall decline in cell growth to below controls (61 and 49%, respectively). When butyrate (1 mmol) was present in the media Ins, IGF-I and EGF overcame the butyrate inhibition, but did not attain the growth observed in uninhibited cultures (67, 57 and 60%, respectively). Although butyrate has been implicated as a possible mediator of ruminal cell growth in vivo, growth factors such as insulin, IGF-I and EGF are more effective inducers of growth in vitro. 

*Keywords:* Rumen; Epithelium; IGF-I; Insulin; Epidermal growth factor

## 1. Introduction

Ruminal papillae size increase markedly with increasing dietary energy intake (Mayer et al., 1986). Consequently, diet formulation changes greatly influence the surface area available for the absorption of nutrients. Changes in surface area are thought to occur in response to alterations in the ruminal VFA concentrations, with high concentrate diets resulting in increased papillae growth (Rickard and Ternouth, 1965). Ruminal concentration of butyrate appears to be the luminal factor that most significantly triggers the growth of ruminal epithelium in vivo (Sakata and Tamate, 1976, 1978). Rapid transient increases of VFA (butyrate, propionate and acetate) in the ruminal lumen result in increased cell mitotic indices of sheep

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in vivo (Sakata and Tamate, 1978), with butyrate being the most efficacious at stimulating ruminal cell proliferation. In contrast, butyrate treatment of rumen epithelial cells as well as other cell lines in vitro result in decreased cell DNA synthesis (Kruh, 1982; Neogrady et al., 1989). Presumably, the disparate actions of butyrate in vivo and in vitro are due to a mechanism involving the metabolism of butyrate in vivo and consequently, a hormonal response to the metabolites of butyrate (Galfi et al., 1991). Several hormones have been implicated as probable mediators of the butyrate-induced stimulation of ruminal epithelial proliferation in vivo which have been reviewed previously by Galfi et al. (1991) and include insulin (Neogrady et al., 1989), pentagastrin, glucagon, and cortisol (Galfi et al., 1991). These hormones are stimulatory to ruminal cell growth in vitro and insulin has also been shown to be stimulatory to ruminal cell mitotic indices when administered in vivo via intravenous infusion (Sakata et al., 1980).

Numerous growth factors, which are produced and act locally within epithelial tissues, must also be considered as possible mediators of the butyrate-induced stimulation of rumen epithelial cell proliferation observed in vivo. Epidermal growth factor (EGF) has been implicated as a proliferative agent in numerous tissues, including rat gastric and intestinal mucosa (Tarnawski et al., 1992; Playford et al., 1993), pig jejunal and upper gastrointestinal mucosa (Jaeger and Lamar, 1992; Kelly et al., 1992), and human epidermal keratinocytes (Peter et al., 1993). Similarly, transforming growth factor-$\alpha$, which acts through the EGF receptor, elicits proliferative actions in epithelial cell lines and tissues including rat oral ketatinocytes, rat gastric tissues, human keratinocytes and mouse skin (Staliano-Coico et al., 1990; Tarnawski et al., 1992; Vassar et al., 1992; Donnelly et al., 1993; Odze et al., 1993). EGF receptors are present in gastrointestinal tissues and keratinized tissues of many species including the pig, rat, and mouse (Odze et al., 1993; Orsini et al., 1993; Peter et al., 1993; Wang et al., 1994). Insulin-like growth factor-I (IGF-I) elicits cell-proliferative effects in mammary epithelial cells (Fenton and Sheffield, 1994) and enhances migration of human keratinocyte cultures (Fenton and Sheffield, 1994). The objectives of this study were to determine the proliferative response of isolated ruminal cells in culture to butyrate, propionate, insulin, IGF-I, and EGF and to determine if butyrate altered the responsiveness of the cells to various agents.

## 2. Materials and methods

### 2.1. Reagents

Trypsin (1:250), Dulbecco's Modified Eagle Medium (DMEM), Hanks' buffered salt solution (Hanks) and *N*-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES; free acid) from GIBCO/BRL (Life Technologies, Gaithersburg, MD 20877) were used. Rehatuin Fetal Bovine Serum (FBS) was purchased from Intergen (Purchase, NY 10577). Long $R^3$ insulin-like growth factor-I (IGF-I; Recombinant analog of human IGF-I) was obtained from GroPep. (Adelaide SA 5000, Australia). Human recombinant EGF was purchased from Upstate Biotechnology (Lake Placid, NY 12946). MTT (3-[4, 5, Dimethylthialzol-2-yl]-2, 5, diphenyltetrazolium bromide; Thiazolyl blue; $C_{18}H_{16}N_5SBr$), insulin (bovine pancreas) and all other reagents were from Sigma (St. Louis, MO 20877).

### 2.2. Rumen cell isolation

Animal care and tissue isolation work were conducted in accordance with animal use protocol # 94-058 reviewed and approved by the Beltsville Area Animal Care and Use Committee, June 1994. Sheep (5 mixed breed ram lambs, aged 2–6 months) were stunned with a captive bolt gun, and rapidly exsanguinated. Rumen tissue and cell isolation procedures were conducted as described previously (Baldwin and Jesse, 1991). Briefly, the ventral cranial sac of the rumen was excised, rinsed and immediately placed in an isotonic wash buffer (Krebs salts, plus 25 mmol HEPES, pH 7.4; KSHEPES) at 37°C for transport to the laboratory. Ruminal epithelium was separated from underlying musculature and minced with scalpels. The tissue was maintained at 37°C in oxygenated KSHEPES throughout the isolation process. Approximately 10 g (wet weight) of papillae were added to 50 ml digestion solution (2% trypsin and 1.15 mmol $CaCl_2$ in KSHEPES) and placed in an orbital hot air incubator at 37°C. After the papillae had undergone tryptic digestion for 15 min, the solution (fraction 1) was filtered through a 300 μm nylon mesh into 50 ml

centrifuge tubes. Rumen epithelial fragments remaining on the mesh were rinsed thoroughly with KSHEPES and the rinse added to the above filtrate. Rumen epithelial fragments generally underwent 5–6 cycles of digestion with fresh trypsin solution (15 min per cycle), depending upon the degree of ruminal cornification and the apparent cell yields.

Following filtration, cell fractions were centrifuged at $60 \times g$ for 4 min at 4°C to pellet the rumen cells. Cell pellets were resuspended in KSHEPES. Cells from the third through final digestion fractions were pooled and washed three times as described above. These later fractions are enriched for the cells of the stratum spinosum and stratum basale ( Galfi et al., 1980; Baldwin and Jesse, 1991), with declining numbers of the terminally differentiated cells of the stratum corneum. Cells were then subjected to four more wash cycles in sterile Hanks' buffered salt solution (Hanks) with antibiotic-antimycotic (100 units $ml^{-1}$ Penicillin G sodium, 100 units $ml^{-1}$ streptomycin sulfate, 0.25 μg $ml^{-1}$ amphotericin B as Fungizone). Cells were counted using a hemacytometer and cell viabilities were estimated by trypan blue dye exclusion. Cells were then plated on plastic 100 mm plates at a density of 1 million cells/plate in DMEM with antibiotic-antimycotic and 5% fetal bovine serum (DMEM-FBS). Following 24 h in culture cell media were removed and replaced with fresh DMEM-FBS. Cell media were changed every 48 h until the cells reached confluence (4–7 days). When the cells had reached confluence they were removed from the plate by trypsinization, quantified and replated on 100 mm plates for culturing, 96-well tissue culture plates for treatment or frozen in a biofreezer for later use.

### 2.3. Growth assays and statistics

Five separate cell preparations were used to assess the effects of growth factors and VFA on ruminal cell growth. Cells from three animals were used in experiments following the first through fifth passage while cells from two animals were used at passages nine through 13. For treatments, 96-well microtiter plates were seeded at $1 \times 10^5$, $2 \times 10^5$, or $2.5 \times 10^5$ cells $well^{-1}$ for 24 h in DMEM-FBS (2.5% FBS). Cells were then serum-starved in DMEM for an additional 24 h prior to the addition of treatments. Negative control wells were refed with DMEM devoid of serum and positive controls were treated with DMEM-FBS (5% FBS). Treatments were administered in 150 μl DMEM distributed randomly to wells at the following concentrations: EGF (0.0167, 0.167, 1.6667, 8.3, 16.67, 83.3 nmol $l^{-1}$), insulin (0.0087, 0.087, 0.174, 0.872, 8.72, 87.2 nmol $l^{-1}$), insulin-like growth factor-I (IGF-I; 0.065,.65, 6.5, 13, 65, 130 nmol $l^{-1}$), butyrate (0.1, 1, 5, 10, 50, 100 mmol $l^{-1}$), and propionate (0.1, 1, 10, 25, 50, 100 mmol $l^{-1}$).

Cell growth following 48 h in treatment media was determined by an MTT-based bio-assay as described by the manufacturer. Briefly, all media on treated 96-well plates were removed, and MTT solution (0.5 mg $ml^{-1}$ in 0.85% saline) was added at 50 μl $well^{-1}$. Following a 6 h incubation, isopropanol solution (50 ml isopropanol, 0.33 ml 6 M HCl, and 25 ml 0.85% saline), was added at 150 μl $well^{-1}$, and the entire solution was mixed using a multi-dispensing pipette to dissolve the formazan crystals which form as a result of mitochondrial dehydrogenase activity present in viable cells. A Ceres900HDi Microtiter Plate Reader (Biotek Instruments) was used to determine absorbance at a wavelength of 565 nm. Absorbance data were used to calculate relative ruminal cell growth with treatment for 48 h.

Absorbance data were used to develop the parameter estimates for a non-linear asymptotic growth curve by PROC TNLIN of SAS. The equation used was:

$$Y = a - (a - b) \times 2^{-x/c}$$

in which

- $Y$ absorbance (at 565 nm) or ruminal cell growth,
- $a$ asymptotic limit of absorbance or ruminal cell growth,
- $b$ absorbance or ruminal cell growth when dose = 0,
- $c$ dose of growth factor at half maximal response,
- $x$ dose of growth factor or VFA.

Parameter estimates were obtained for each animal and differences were determined by analysis of variance by PROC GLM of SAS (SAS Institute) where growth of cells in the presence of 5% FBS was included as a covariate. Differences were considered significant at $p < 0.05$.

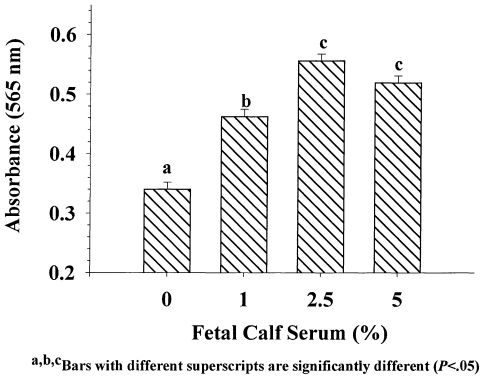


Fig. 1. Proliferative response of cultured rumen epithelial cells with increasing concentrations of fetal bovine serum in the media. Ruminal cells were incubated in DMEM in the presence of either 0, 1, 2.5, or 5% FBS for 48 h. Bars represent the mean ± SEM absorbance at 565 nm of at least 3 animals. [a,b,c]Bars with different superscripts are significantly different ($p < 0.05$).

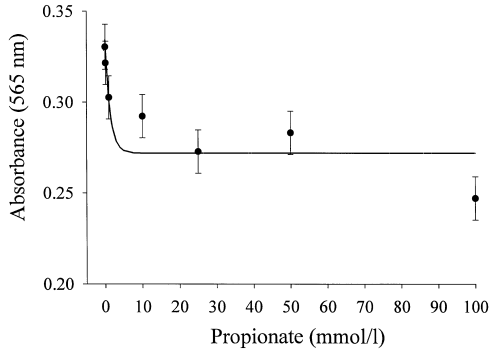


Fig. 2. Proliferative response of cultured rumen epithelial cells with increasing concentrations of propionate. Ruminal cells were incubated in DMEM in the presence of propionate for 48 h. Symbols and error bars represent the mean ± SEM absorbance at 565 nm of observed data from at least 3 animals.

## 3. Results and discussion

Optimal FBS supplementation for the isolated ruminal cells was determined by incubation of cells in DMEM supplemented with 0, 1, 2.5, and 5% FBS for 48 h and cell proliferation was determined by MTT assay. Cell growth was stimulated ($p < 0.05$) at all serum additions with maximal response at 2.5% FBS supplementation; however, 2.5% FBS supplementation was not significantly different than the 5% FBS treatment (Fig. 1). All subsequent experiments used 5% FBS as a positive control for cell proliferation.

Treatment of rumen epithelial cells in culture with propionate resulted in decreased cell proliferation in a dose-dependent fashion (Fig. 2). The half-maximal inhibitory concentration ($IC_{50}$), lower asymptote of response and the intercept at no propionate addition are presented in Table 1. Half-maximal inhibitory response in the present study is consistent with the findings of Neogrady et al. (1989) of an $IC_{50}$ of 1.47 mmol $l^{-1}$. Maximal inhibition by propionate represents a 61% decrease (absorbance corrected for negative control/absorbance change due to 5% FBS

Table 1
Parameter estimates for growth curve predictions of ruminal cell proliferative response to various treatments

| Addition | Asymptote[a] | | Intercept[a] | | Half-maximal response[b] | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE |
| Propionate | 0.27 | 0.009 | 0.33 | 0.013 | 0.93 | 0.828 |
| Butyrate | 0.26 | 0.009 | 0.33 | 0.014 | 0.52 | 0.461 |
| Insulin | 0.47 | 0.022 | 0.34 | 0.012 | 2.46 | 1.995 |
| Insulin plus butyrate[c] | 0.46 | 0.019 | 0.36 | 0.013 | 1.99 | 2.208 |
| IGF-I | 0.51 | 0.023 | 0.36 | 0.022 | 2.62 | 2.211 |
| IGF-I plus butyrate[c] | 0.47 | 0.016 | 0.35 | 0.024 | 0.57 | 0.480 |
| EGF[d] | 0.52 | 0.013 | 0.38 | 0.012 | 1.87 | 0.911 |
| EGF plus butyrate[c] | 0.43 | 0.013 | 0.31 | 0.013 | 1.47 | 0.782 |

[a] Absorbance at 565 nm following MTT assay as described in Section 2.
[b] Concentration of addition at which half-maximal response was attained in mmolar (propionate and butyrate) and nmolar (Insulin, IGF-I and EGF).
[c] Butyrate concentration was constant at 1 mmol $l^{-1}$.
[d] Epidermal growth factor.

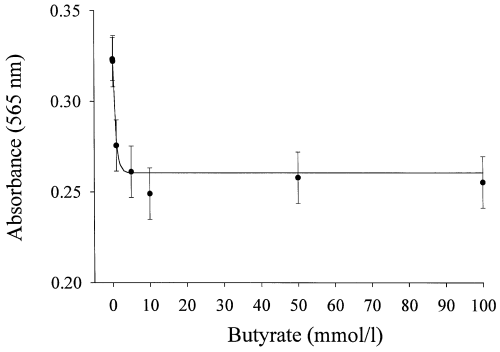


Fig. 3. Proliferative response of cultured rumen epithelial cells with increasing concentrations of butyrate. Ruminal cells were incubated in DMEM in the presence of butyrate for 48 h. Symbols and error bars represent the mean ± SEM absorbance at 565 nm of observed data from at least 3 animals.

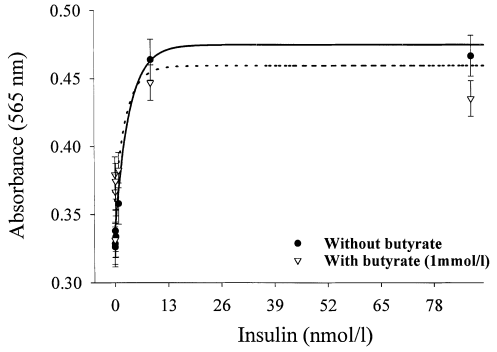


Fig. 4. Proliferative response of cultured rumen epithelial cells with increasing concentrations of insulin in the presence and absence of butyrate. Ruminal cells were incubated in DMEM in the presence of insulin with or without butyrate (1 mmol $l^{-1}$) for 48 h. Symbols and error bars represent the mean ± SEM absorbance at 565 nm of observed data from at least 3 animals.

addition) in cells following 48 h incubations. These results are consistent with studies by others (Galfi et al., 1981, 1991) where increasing concentrations of various VFA and metabolic end-products of butyrate (ketones) were tested and propionate was shown to inhibit $^3$H-Thymidine incorporation by ruminal cells in primary culture. In these studies, the increasing chain length of the metabolites was correlated with increased inhibition of $^3$H-Thymidine incorporation. Butyrate treatment in the present study resulted in inhibition of cell proliferation, relative to the controls, as was expected (Fig. 3). The half-maximal inhibition achieved in 48 h incubations was 0.52 mmol $l^{-1}$ butyrate (Table 1). Neogrady et al. (1989) reported 50% inhibitory concentrations of butyrate ranging from 0.28 to 0.33 mmol $l^{-1}$ by $^3$H-Thymidine incorporation and cell counts, respectively. Maximal inhibition by butyrate resulted in a 49% decline in absorbance relative to positive controls (5% FCS stimulated). This is contradictory to the findings of Neogrady et al. (1989), in that butyrate inhibition was not as strong as that observed for propionate. This could be a result of the higher doses used in the present study (100 mmol as compared to 5 mmol). There was significant animal-to-animal (cell-preparation-to-cell-preparation) variation observed in this study and in one animal the observed $IC_{50}$ for propionate was 6 mmol $l^{-1}$. Therefore, at high concentrations it appears that the effect of increasing chain length is not as clear. Conceivably, because metabolic conversion of butyrate to ketones is an important function of ruminal metabolism, the cells are able to survive better in elevated butyrate concentrations through metabolic conversion of the butyrate to less toxic metabolites.

Insulin stimulation of 48 h ruminal cell proliferation is dose-dependent with half maximal stimulation ($ED_{50}$), occurring at 2.4 nmol concentrations (Fig. 4 and Table 1). Insulin concentrations of 0.16–1.6 nmol $l^{-1}$ used in studies by Neogrady et al. (1989) induced a proliferative response; however, an $ED_{50}$ was not reported. The $ED_{50}$ in the present study (Table 1) was higher than the concentrations tested by Neogrady et al. (1989), which when combined with differences in the procedures used, makes comparison of the entire dose response of ruminal cell proliferation difficult. The protocol in the present study consisted of a negative control devoid of serum following a 24 h serum-starved pretreatment, while the studies by Neogrady et al. (1989) were conducted in the presence of serum which contained 0.9 μU $ml^{-1}$ of insulin. Regardless, insulin elicits a stimulatory response in the nmolar range resulting in a maximal response of 75% of the positive controls (5% FBS-supplemented DMEM). When both increasing doses of insulin and 1 mmol $l^{-1}$ butyrate were included in the media, insulin overcame in part the inhibitory action of butyrate (Fig. 4). At higher concentrations of insulin, butyrate-treated cells were unable to grow at the rate observed when incubated with insulin alone ($p < 0.05$). Although the maximal response was not

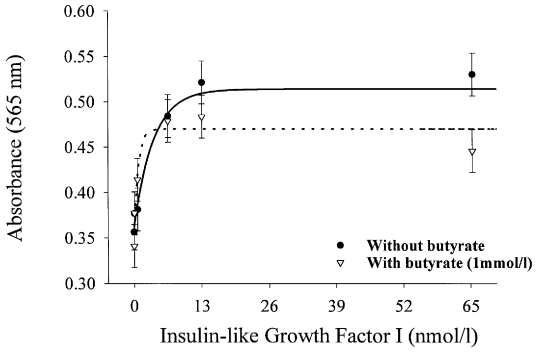


Fig. 5. Proliferative response of cultured rumen epithelial cells with increasing concentrations of insulin-like growth factor-I in the presence and absence of butyrate. Ruminal cells were incubated in DMEM in the presence of insulin-like growth factor-I with or without butyrate (1 mmol $l^{-1}$) for 48 h. Symbols and error bars represent the mean ± SEM absorbance at 565 nm of observed data from at least 3 animals.

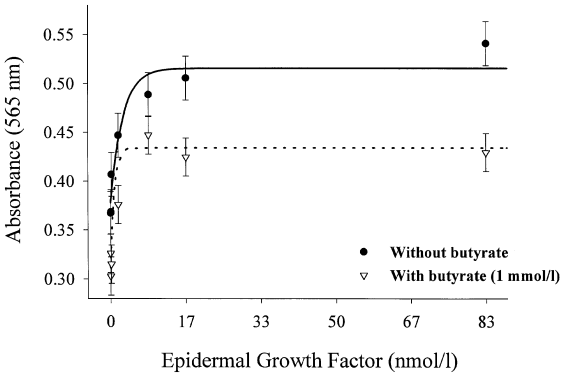


Fig. 6. Proliferative response of cultured rumen epithelial cells with increasing concentrations of EGF in the presence and absence of butyrate. Ruminal cells were incubated in DMEM in the presence of EGF with or without butyrate (1 mmol $l^{-1}$) for 48 h. Symbols and error bars represent the mean ± SEM absorbance at 565 nm of observed data from at least 3 animals.

attained in the presence of butyrate (67 vs. 75% of positive controls) the magnitude of the change was the same as observed in the absence of butyrate (low dose to high dose). Furthermore, the dose required to attain half-maximal response was not changed by butyrate addition (Table 1). Therefore, it appears that the inhibition induced by butyrate is consistent, regardless of the stimulation of insulin.

Insulin-like growth factor-I addition stimulated ruminal cell growth to levels which matched the proliferative effects of serum (96% of positive controls; Fig. 5). Similar to insulin, IGF-I achieved half-maximal stimulation at 2.6 nmol $l^{-1}$. In contrast to insulin, although butyrate (1 mmol $l^{-1}$) addition resulted in decreased ruminal cell proliferation across all doses tested (69% of the positive control at maximal response), the concentration necessary to induce a half-maximal stimulation is numerically reduced (2.6 vs. 0.57 nmol $l^{-1}$ IGF-I, alone and with 1 mmol butyrate addition, respectively; $p < 0.2$). The $ED_{50}$ was consistently lower in the butyrate-treated ruminal cells; however, large animal-to-animal variations in the parameter estimates resulted in high standard error estimates. Further investigations are necessary, with more concentrations in the 0.1–10 nmol range used, to establish if butyrate reduced the $ED_{50}$ of IGF-I. Conceivably, butyrate could induce differential gene expression by the ruminal cells which results in an increased sensitivity to the growth factors present in the extracellular milieu. Alternatively, butyrate treatment of primary cultures could be differentially affecting the cell-types that make up the culture. Cells could be selected for their resilience to the inhibitory actions of butyrate and these may be the cells which are most capable of responding to growth factors.

EGF also stimulated ruminal cell proliferation in primary cultures of rumen epithelial cells (Fig. 6). Ruminal cells treated with EGF attained a maximal growth not significantly different than the positive controls (97 of the 5% FBS-treated cell growth). The inhibitory action of 1 mmol $l^{-1}$ butyrate was overcome in part by the addition of EGF, but even at high EGF concentrations butyrate treatment reduced the proliferative response to 60% of that achieved in the positive controls. Similar to the insulin response, at all concentrations of EGF tested, the addition of butyrate reduced cell proliferation. The $ED_{50}$ for EGF decreased numerically by butyrate treatment but was not significantly reduced, again, due to animal-to-animal variation. As for the results with IGF-I, additional experiments need to be conducted to further elucidate the actions of butyrate on the rumen epithelial cells' interaction with growth factors.

When the dietary regime, either through change in diet or feeding pattern, is altered the environment in the ruminal liquor is altered. These alterations may require that the ruminal epithelium respond to injuries

incurred as a result of dramatic changes in the pH and end-products of fermentation that are potentially harmful to the animal. EGF is also of interest due to its capacity to alter keratin expression in keratinocytes (Cheng et al., 1993; Jiang et al., 1993). The expression of these growth factors by epithelial cells is upregulated in response to injury induced by chronic alcohol administration in rats (Tarnawski et al., 1992), allergic and inflammatory responses and radiation-induced injury of epidermal keratinocytes (Jiang et al., 1993; Peter et al., 1993). Butyrate treatment, while inhibiting cell proliferation, results in increases in cell size and protein synthesis in HELA cells (Galfi et al., 1985), and has differential effects on keratinized and non-keratinized rumen epithelial cells in culture. Therefore, it is possible that, by altering cellular response to direct actions at the ruminal epithelium, butyrate affects cell proliferation and differentiation through local growth factors such as EGF and IGF-I. The ruminal epithelium performs numerous vital functions for the ruminant, including protection, absorption and metabolism. Due to the need for intensive agriculture and high-concentrate feeding regimes, there is a need for an increased understanding of the impact of these regimes on ruminal metabolism and the health of the ruminal epithelium. The effect of butyrate is well documented; however, the mechanisms by which this action is mediated are not completely defined. The possible role of growth factors, and their interaction with butyrate, are not well understood. It appears that insulin-like growth factor-I and EGF and, to a lesser extent insulin, may play a crucial role in the health and metabolism of the ruminal epithelium.

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